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(57) Abstract

The present invention discloses a novel enzyme which is an adipoyl-cocenzyme A synthetase. Further the invention relates to a DNA fragment encoding said novel enzyme, an expression cassette, recombinant vector or transformation vehicle comprising said DNA fragment and finally a cell comprising said expression cassette or recombinant vector. In addition, the invention relates to the use of the novel enzyme in processes for producing β-lactam antibiotics.
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An enzyme with high adipoyl-coenzyme A synthetase activity and uses thereof

Field of the invention

The present invention relates to a novel enzyme which advantageously can be used in the β-lactam antibiotic biosynthesis. Further the invention relates to a DNA construct encoding said novel enzyme, a recombinant vector or transformation vehicle comprising said DNA construct, and finally a cell comprising said DNA construct or recombinant vector. In addition, the invention relates to processes for producing β-lactam antibiotics.

Background of the invention

β-Lactam antibiotics constitute the most important group of antibiotic compounds, with a long history of clinical use. Among this group, the prominent ones are the penicillins and cephalosporins. These compounds are naturally produced by the filamentous fungi Penicillium chrysogenum and Acremonium chrysogenum, respectively.

As a result of classical strain improvement techniques, the production levels of the antibiotics in P. chrysogenum and A. chrysogenum have increased dramatically over the past decades. With the increasing knowledge of the biosynthetic pathways leading to penicillins and cephalosporins, and the advent of recombinant DNA technology, new tools for the improvement of production strains and for the in vivo derivatization of the compounds have become available.

Most enzymes involved in β-lactam biosynthesis have been identified and their corresponding genes been cloned, as is described by Ingolia and Queener, Med. Res. Rev. 9 (1989), 245-264 (biosynthesis route and enzymes), and Aharonowitz, Cohen, and Martin, Ann. Rev. Microbiol. 46 (1992), 461-495 (gene cloning).

The first two steps in the biosynthesis of penicillin in P. chrysogenum are the condensation of the three amino acids L-5-amino-5-carboxypentanoic acid (L-α-aminoadipic acid) (A), L-cysteine (C) and L-valine (V) into the tripeptide LLD-ACV, followed by cyclization of this tripeptide to form isopenicillin N. This compound contains the typical β-lactam structure.

These first two steps in the biosynthesis of penicillins are common in penicillin-, cephamycin- and cephalosporin-producing fungi and bacteria.
The third step involves the exchange of the hydrophilic α-amino adipic acid side chain derived from L-5-amino-5-carboxypentanoic acid by a hydrophobic side chain, by the action of the enzyme acyltransferase (AT). The enzymatic exchange reaction mediated by AT takes place inside a cellular organelle, the microbody, as has been described in EP-A-0448180.

The only directly fermented penicillins of industrial interest are penicillin V and penicillin G, produced by adding phenoxyacetic acid or phenylacetic acid, respectively, during fermentation of *P. chrysogenum*, thereby replacing the side chains of the natural β-lactams with phenoxyacetic acid or phenylacetic acid.

In order to replace the α-amino adipic acid side chain in the acyltransferase catalyzed reaction, the carboxylic acid group of the new side chain has to be activated, since the AT enzyme requires the side chain in an activated form. The activated form is a coenzyme A (CoA) derivative, synthesized by a CoA ligase.

The gene encoding an acetyl-coenzyme A synthetase of *P. chrysogenum* has been characterized by Van Hartingsveldt *et al.* (WO92/07079), Gouka *et al.* (Appl. Microbiol. Biotechnol., 38, 514-519, 1993) and Martínez-Bianco *et al.* (Gene, 130, p. 265-270, 1993). The gene codes for a polypeptide of 669 amino acids, corresponding to a molecular weight of approximately 74,000 Dalton. The enzyme not only accepts acetic acid but also phenylacetic acid as a substrate in the synthesis of the corresponding acyl-coenzyme A ester, although the specificity for phenylacetic acid is very low as compared to the specificity for acetic acid.

In novel fermentation processes for the production of cephalosporin intermediates, like 7-aminodeacetoxycephalosporanic acid (7-ADCA), 7-aminodeacetylcephalosporanic acid (7-ADAC) or 7-aminocephalosporanic acid (7-ACA), *P. chrysogenum* strains carrying heterologous cephalosporin biosynthetic genes, like the expandase gene of *S. clavuligerus* or the expandase/hydroxylase gene of *A. chrysogenum*, are used for the production of the corresponding adipoylcephalosporins by feeding adipic acid to these recombinant strains. Subsequent removal of the adipoyl side chain from the adipoylcephalosporins can be achieved by using an amidase (EP 532341 and Crawford *et al.*, Bio/Technology 13, p. 58-62, 1995).

Although β-lactam yields are high in industrial *P. chrysogenum* strains, yields of adipoylcephalosporins are still low, since *P. chrysogenum* penicillin G production strains are used for the production of said adipoyl-compounds. Penicillin G production strains are optimised for a high yield on the phenylacetic acid side chain precursor and consequently a low level of β-lactam byproducts lacking said side chain, like 6-aminopenicillanic acid (6-APA) and 8-OH penicillic acid.
However, relatively high levels of 6-APA, 8-OH penicillic acid and other byproducts are detected in adipate fed fermentations. To eliminate or reduce the formation of these byproducts and to increase the yield of adipylcephalosporins, the formation of adipoyl-6-APA and adipoyl-7-ADCA should be optimised.

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Summary of the invention

The present invention discloses a novel enzyme which is an acyl-coenzyme A synthetase, more specifically an adipoyl-coenzyme A synthetase.

According to the present invention, it has been shown that said acyl-coenzyme A synthetase is able to catalyze the formation of adipoyl-coenzyme A with high efficiency. In addition, said acyl-coenzyme A synthetase is able to catalyze the formation of other acyl-coenzyme A compounds, for instance adipoyl-coenzyme A derivatives such as carboxymethylthiopropionyl-coenzyme A and trans-β-hydromuconyl-coenzyme A.

The enzyme of the invention is at least between 10 and 100 times, preferably 10^3 times and more preferably between 10^4 and 10^5 times more active towards adipic acid than towards acetic acid or than towards phenylacetic or phenoxyacetic acid.

According to the invention, the enzyme is obtainable from *Penicillium chrysogenum*.

The novel enzyme can be used in connection with biosynthesis of various β-lactam antibiotics.

Also contemplated is a DNA fragment comprising a DNA sequence encoding said novel enzyme exhibiting adipoyl-coenzyme A synthetase activity, and an expression cassette comprising said DNA sequence.

It is a further object of the invention to provide a vector or transformation vehicle comprising said expression cassette.

Also contemplated according to the invention is a microbial cell comprising said expression cassette or said vector or transformation vehicle.

Detailed description of the invention

The present invention discloses a novel enzyme which is an acyl-coenzyme A synthetase, more specifically an adipoyl-coenzyme A synthetase. This enzyme will in the following be referred to as "ligase" or "CoA ligase".

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According to the present invention it has been shown that said ligase is able to catalyze the formation of adipoyl-coenzyme A from Mg\(^{2+}\), ATP, CoASH and adipic acid with a high efficiency.

It has further been shown that said ligase of the invention is able to catalyze the formation of other acyl-coenzyme A compounds than adipoyl-coenzyme A, e.g. the formation of derivatives of adipoyl-coenzyme A, such as carboxymethylthiopropionyl-coenzyme A and trans-\(\beta\)-hydromuconyl-coenzyme A, from Mg\(^{2+}\), ATP, CoASH and derivatives of adipic acid, such as carboxymethylthiopropionic acid and trans-\(\beta\)-hydromuconic acid, respectively. When assayed in the same system (e.g. as described in the examples below) the ligase does not show any significant activity towards acetic acid.

Thus, the CoA ligase of the invention has a high affinity towards adipic acid as a substrate. The ligase of the invention additionally has an affinity towards other important \(\beta\)-lactam side chain precursors, such as phenoxyacetic acid and phenylacetic acid, albeit substantially lower than towards adipic acid. The CoA ligase of the invention further has a very low specificity towards acetic acid.

More specifically, the novel enzyme, according to the invention, is at least between 10 and 100 times, preferably 10\(^3\) times and more preferably between 10\(^4\) and 10\(^5\) times more active towards adipic acid than towards acetic acid.

Further, the enzyme, according to the invention, is at least between 10 and 100 times, preferably 10\(^3\) times and more preferably between 10\(^4\) and 10\(^5\) times more active towards adipic acid than towards phenylacetic or phenoxyacetic acid.

The enzyme is unstable in vitro, but addition of ATP, Mg\(^{2+}\) and \(\beta\)-mercaptoethanol, dithiothreitol (DTT), ascorbic acid or other reducing agents significantly stabilizes the activity during purification and storage. The presence of a high concentration of ammonium sulphate or glycerol also stabilizes the enzyme.

Only when assayed in the absence of other thiols (e.g. mercaptoethanol, dithiotreitol), a direct adipoyl-coenzyme A synthetic activity was observed. However, incubation of ligase with ATP, Mg\(^{2+}\), CoASH, adipic acid and e.g. mercaptoethanol leads to the formation of a compound which has the same UV-spectrum as the thioester of adipic acid and mercaptoethanol, adipoyl-S-CH\(_2\)-CH\(_3\), which also can act as a substrate for the acyltransferase (AT).

In the present context enzymes according to the invention include mature proteins or precursor forms thereof and functional fragments thereof which essentially have the activity of the full-length polypeptides.

Further contemplated according to the invention are homologues of said enzymes. Such homologues comprise an amino acid sequence exhibiting a degree
of identity of at least between 50% and 70%, better between 70% and 80%, even better up to 100%, with the amino acid sequence of the enzyme according to the present invention.

The degree of identity may be determined by conventional methods, see for instance: Altshui et al. (Bull. Math. Bio., 48, 603-616, 1986) and Henikoff and Henikoff (Proc. Natl. Acad. Sci. USA, 89, 100915-10919, 1992). Briefly, two amino acid sequences are aligned to optimize the alignment scores using a gap opening penalty of 10, a gap extension penalty of 1, and "blosum 62" scoring matrix of Henikoff and Henikoff, supra.

Alternatively, the homologue of the enzyme according to the invention may be one encoded by a nucleotide sequence hybridizing with an oligonucleotide probe prepared on the basis of the nucleotide sequence of said enzyme exhibiting ligase activity.

Molecules to which the oligonucleotide probe hybridizes under these conditions are detected using standard detection procedures (e.g. PCR technology, Southern blotting).

Homologues of the present polypeptide may have one or more amino acid substitutions, deletions or additions. These changes are preferably of a minor nature, that is conservative amino acid substitutions that do not adversely affect the folding or activity of the protein, small deletions, typically of one to about 30 amino acids; small amino- or carboxyl-terminal extensions, such as an amino-terminal methionine residue, a small linker peptide of up to about 20-25 residues, or a small extension that facilitates purification, such as a poly-histidine tract, an antigenic epitope or a binding domain. See in general Ford et al. (Protein Expression and Purification, 2, 95-107, 1991). Examples of conservative substitutions are within the group of basic amino acids (such as arginine, lysine, histidine), acidic amino acids (such as glutamic acid and aspartic acid), polar amino acids (such as glutamine and asparagine), hydrophobic amino acids (such as leucine, isoleucine, valine), aromatic amino acids (such as phenylalanine, tryptophan, tyrosine) and small amino acids (such as glycine, alanine, serine, threonine, methionine).

It will be apparent to persons skilled in the art that such substitutions can be made outside the regions critical to the function of the molecule and still result in an active enzyme. Amino acids essential to the activity of the enzyme of the invention, and therefore preferably not subject to substitution, may be identified according to procedures known in the art, such as site-directed mutagenesis or alanine-scanning mutagenesis (Cunningham and Wells, Science, 244, 1081-1085, 1989). In the latter technique mutations are introduced at every residue in the molecule, and the resultant mutant molecules are tested for biological activity (e.g.
ligase activity to identify amino acid residues that are critical to the activity of the molecule. Sites of ligand-receptor interaction can also be determined by analysis of crystal structure as determined by such techniques as nuclear magnetic resonance, crystallography or photoaffinity labelling. See, for example, de Vos et al. (Science, 255, 306-312, 1992), Smith et al. (J. Mol. Biol., 224, 899-904, 1992) and Wlodaver et al. (FEBS Lett., 309, 59-64, 1992).

The homologue may be an allelic variant, i.e. an alternative form of a gene that arises through mutation, or an altered enzyme encoded by the mutated gene, but having substantially the same activity as the enzyme of the invention. Hence mutations can be silent (no change in the encoded enzyme) or may encode enzymes having altered amino acid sequence.

The homologue of the present enzyme may also be a species homologue, i.e. an enzyme with a similar activity derived from another species.

Further homologues of the present enzyme are those which are immunologically cross-reactive with antibodies raised against the enzyme of the invention.

According to the invention, the ligase is obtainable from bacteria or fungi. Preferably, the ligase is obtainable from the filamentous fungi Penicillium chrysogenum, Aspergillus nidulans or Acrocomium chrysogenum. More preferably, the enzyme is obtainable from Penicillium chrysogenum.

The present invention surprisingly shows that ligases partially purified from cell-free extracts obtained from P. chrysogenum fermentations which are fed by different side chain precursors display an acyl-coenzyme A synthetase activity with a different specificity towards different side chain precursors.

The CoA ligase of the invention specifically accumulates within the cell when performing a fermentation in the presence of an inducing substance, i.e. the relevant side chain precursor, which is adipic acid, or an analogue thereof.

In this way, the expression of the ligase of the invention is specifically increased in respect to other CoA ligases with a different substrate specificity.

Once the CoA ligase having a high affinity for adipic acid is identified, the enzyme can be purified from P. chrysogenum biomass using conventional protein purification techniques.

For instance, the enzyme can be purified from cell extracts of P. chrysogenum by precipitation with ammonium sulphate and by elution from columns packed with various gels (e.g. Phenylsepharose, Cibacron blue and Sephacryl\textsuperscript{S} S-200).

The availability of the pure enzyme enables the skilled person to determine partial amino acid sequences of the protein, from its N-terminus and/or from internal fragments. In case the enzyme preparation is not substantially homogeneous, partial
amino acid sequences can be directly determined from protein or peptide bands separated by electrophoresis of a protein or peptide preparation on a denaturing SDS gel (Matsudaira, Methods Enzymol. 182, 602-613, 1990). The thus-obtained amino acid sequence data subsequently enable the skilled person to construct oligonucleotide probes to be used for cloning of the corresponding gene or cDNA sequence from an appropriate library. Optionally, PCR technology can be used to facilitate cloning.

A DNA fragment comprising a nucleotide sequence encoding the ligase of the invention, may suitably be of genomic or cDNA origin, for instance obtained by preparing a genomic or cDNA library and screening for DNA sequences coding for all or part of the polypeptide by hybridization using synthetic oligonucleotide probes in accordance with standard techniques (cf. Sambrook et al., Molecular Cloning: A Laboratory Manual, 2nd. Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1989).

The DNA fragment comprising the gene or cDNA encoding the ligase of the invention may also be prepared by polymerase chain reaction using specific primers, for instance as described in US 4,683,202 or Saiki et al. (Science, 239, 487-491, 1988).

In a preferred embodiment of the invention, the DNA fragment comprising the DNA sequence encoding the ligase of the invention is derivable from a filamentous fungus belonging to genus of Aspergillus, Penicillium or Acremonium, preferably from a strain of P. chrysogenum, A. chrysogenum, or A. nidulans, more preferably from a strain of P. chrysogenum.

In a further aspect, the present invention relates to an expression cassette comprising said DNA fragment containing a gene or cDNA encoding said enzyme exhibiting ligase activity.

In the expression cassette, the DNA sequence encoding the polypeptide of the invention is operably linked to additional segments required for transcription of the DNA. The term, "operably linked" indicates that the segments are arranged so that they function in concert for their intended purposes, e.g. transcription initiates in a promoter and proceeds through the DNA sequence coding for the polypeptide.

The promoter may be any DNA sequence which shows transcriptional activity in the host cell of choice and may be derived from genes encoding proteins either homologous or heterologous to the host cell.

An overview of fungal promoters can be found in, for instance, Applied Molecular Genetics of filamentous fungi (Kinghorn, Turner (eds.), Blackie, Glasgow, UK, 1992). Suitable promoters for use in filamentous fungus host cells are, for
instance, the ADH3 promoter (McKnight et al., EMBO J., 4, 2093-2099, 1985) or the tpiA promoter. Examples of other useful promoters are those derived from the gene encoding A. oryzae TAKA amylase, Rhizomucor miehei aspartic proteinase, A. niger neutral α-amylase, A. niger acid stable α-amylase, A. niger or A. awamori glucoamylase (gluA), Rhizomucor miehei lipase, A. oryzae alkaline protease, A. oryzae triose phosphate isomerase, A. nidulans acetamidase, P. chrysogenum ACV synthetase, P. chrysogenum isopenicillin N synthase, P. chrysogenum acyltransferase, P. chrysogenum phosphoglycerate kinase, P. chrysogenum gene Y. Preferred are the A. niger glucoamylase or P. chrysogenum promoters.

It is often advantageous to use identical or similar promoters to regulate two or more of the biosynthetic genes in order to obtain a synchronized production of the intermediates involved in the β-lactam antibiotic synthesis. If the production of intermediates is not synchronized an accumulation of intermediates (bottle neck) might arise. Consequently the production of β-lactam antibiotic may be retained.

In an embodiment of the invention the promoter of said ligase gene is replaced by the promoter from another gene involved in the biosynthesis of β-lactams.

Examples of suitable promoters for use in bacterial host cells include the promoter of the Bacillus stearothermophilus maltogenic amylase gene, the Bacillus licheniformis alpha-amylase gene, the Bacillus amyloliquefaciens BAN amylase gene, the Bacillus subtilis alkaline protease gen, or the Bacillus pumilus xylosidase gene, or by the phage Lambda P₆ or P₇ promoters or the E. coli lac, trp or tac promoters.

The DNA fragment encoding the enzyme of the invention may also, if necessary, be operably connected to a suitable terminator.

To direct a ligase enzyme of the present invention to the desired location within the host cell or into the fermentation media, a targeting signal or a secretory signal sequence (also known as a leader sequence, prepro sequence or pre sequence), respectively, may be provided in the expression cassette. The targeting signals or secretory signal sequence are joined to the DNA sequence encoding the enzyme in the correct reading frame. Secretory sequences are commonly positioned 5’ to the DNA sequence encoding the enzyme, whereas the targeting signal sequences are commonly positioned 3’ to the DNA sequence. The targeting signal or secretory signal sequences may be a sequence natively associated with the gene or may be from a gene encoding another protein having the desired signal sequence.

According to the present invention the targeting sequence is preferably a targeting signal capable of directing the ligase activity to the desired location within the cell (e.g. to the microbodies).
For the use in filamentous fungi, an example of a peroxisomal targeting signal (PTS), with the ability to translocate cytosolic passenger proteins to the peroxisomes (classified as microbodies), in e.g. Neurospora crassa, is described by Keller et al. (J. Cell Biol., 114, 893-904, 1991). For an extensive review of peroxisomal targeting signals see Subramani (Annu. Rev. Cell. Biol. 9, 445-478, 1993).

The expression cassette comprising a DNA fragment containing the gene or cDNA encoding said enzyme exhibiting ligase activity may be incorporated in a recombinant vector or transformation vehicle. The vector into which the expression cassette of the invention is inserted may be any vector which may conveniently be subjected to recombinant DNA procedures, and the choice of the vector will often depend on the host cell into which it is to be introduced. Thus, the vector may be an autonomously replicating vector, i.e. a vector which exists as an extrachromosomal entity, the replication of which is independent of chromosomal replication, e.g. a plasmid. Alternatively, the vector may be one which, when introduced into a host cell, is integrated into the host cell genome and replicated together with the chromosome(s) into which it has been integrated.

The recombinant vector may also comprise a selectable marker, e.g. a gene the product of which complements a defect in the host cell, such as the gene coding for dihydrofolate reductase (DHFR) or the Schizosaccharomyces pombe TPI gene (described by P.R. Russell, Gene, 40, 125-130, 1985), or one which confers resistance to a drug, e.g. ampicillin, kanamycin, tetracyclin, phleomycin, chloramphenicol, neomycin, hygromycin or methotrexate. For filamentous fungi, additional selectable markers include amdS, pyrG, argB, niaD, facA, and sC (Applied Molecular Genetics of Filamentous fungi (ibid.), Biotechnology of Filamentous fungi, Finkelson, Ball (eds.), Butterworth-Heinemann, Boston, 1992).

It is also possible to introduce the expression cassette comprising the DNA sequence encoding the ligase of the invention into a host cell separately from a vector comprising a selectable marker, by a so-called co-transformation process.

According to the invention, several options exist for the improvement of fermentative production of β-lactam compounds in which adipic acid or other side chain precursors are fed.

For instance, such improved processes can be provided if the production of β-lactam antibiotics of interest takes place in the presence of increased ligase activity.

According to the invention, such improved process of producing a β-lactam antibiotic, comprises

i) fermentation of a microorganism capable of producing said β-lactam antibiotic, and
retrieving said β-lactam antibiotic in substantially pure form,
wherein said fermentation takes place in the presence of increased ligase activity,
in comparison to the ligase activity present when fermenting with the original
microorganism alone and/or under the original fermenting conditions.

An increased ligase activity is defined as an enhanced conversion of the
carboxylic acid in question towards the corresponding acyl-coenzyme A ester, in
comparison to the unmodified original microorganism and/or the original fermentation
conditions. Said original microorganism, capable of producing β-lactams, lacks or
only has a relatively low ligase activity and/or said original fermentation conditions
do not give any or result in only a relatively low ligase expression.

The increased expression of ligase activity may be accomplished by any
suitable way.

As an example, according to an embodiment of the invention, the ligase
activity may be increased by modulation of the physical conditions of the
fermentation process, such as temperature and pH. Another possibility is subjecting
the microorganism to compounds or agents, leading to an increased expression
of ligase. The nature of said compound or agent depends on e.g. the promoter used
for initiating the expression of the ligase. Further, by interfering with the cellular
control mechanisms controlling the ligase expression, increased expression of ligase
activity can be achieved.

In an embodiment of the invention said ligase activity or increased ligase
activity is obtained by modifying said microorganism.

This can be done by well known procedures, such as introducing at least
one copy of an expression cassette or a recombinant vector, comprising a DNA
fragment encoding said ligase activity, into said original microorganism to be
fermented. The introduction of said expression cassette or recombinant vector into
a host cell may the performed according to, for instance, Applied Molecular Genetics
of Filamentous fungi (ibid.) or Biotechnology of Filamentous fungi (ibid.).

It is also possible that the ligase activity or increased ligase activity is
obtained by random or site specific mutagenesis of said microorganism.

Furthermore, a modification leading to increased ligase activity, may be
obtained by amino acid substitutions, deletions or additions of the ligase enzyme.

The DNA fragment encoding the ligase of the invention may thereby be
derived from a species which is similar to or different from the microorganism in
which it is to be introduced.

In an embodiment of the invention, the DNA fragment encoding the ligase
of the invention is used to delete its genomic counterpart. This may be important
when the ligase is not expressed at the proper location inside the cell. Upon deletion
of the gene, it is possible to introduce the DNA fragment encoding the ligase of the invention provided with the appropriate targeting signals.

In an embodiment of the invention, the DNA fragment encoding the ligase of the invention is used to delete a gene encoding an isozyme of said ligase, i.e. a mitochondrial enzyme.

In certain cases it is desirable to move the entire biosynthesis of a β-lactam, including the expression of the enzyme exhibiting the ligase activity to another microorganism, which is not itself capable of producing said β-lactam. This may be the case if the recipient (host) microorganism e.g. 1) is easier transformed, 2) is able to express the biosynthetic enzymes at high levels, 3) has better growth characteristics or 4) produces less impurities, which may interfere with the recovery. Thus, a higher overall yield of the β-lactam may be obtained.

Alternatively, new biosynthetic routes may be desirable in an organism. According to the invention, the above mentioned recipient or host microorganism is from the group comprising Penicillium, Cephalosporium, Aspergillus, Nocardia, Streptomyces, Bacillus, Cerospora, Microspora, other Eubacteria, other Actinomyces or filamentous fungi.

In a preferred embodiment said microorganism belongs to species from the group comprising Penicillium chrysogenum, Penicillium notatum, Acremonium chrysogenum, Aspergillus nidulans, Nocardia lactamurans and Streptomyces clavuligerus.

In an embodiment of the invention the expression of said ligase activity is synchronized to the expression of other genes belonging to the β-lactam biosynthetic pathway. Said genes may e.g. be the pcbAB, pcbC and/or penDE genes.

The above mentioned β-lactam antibiotic is selected from the group comprising penicillins, cephalosporins, cephamycins.

Preferably said antibiotic is a penicillin or a cephalosporin, more preferably an adipoyl-penicillin or an adipoyl-cephalosporin, such as adipoyl-7-ADCA, adipoyl-7-ADAC or adipoyl-7-ACA.

In an embodiment of the invention the culturing of said microorganism takes place under conditions inducing expression of the DNA sequence encoding the ligase of the invention, thus resulting in an increased production of the coenzyme A thioester of the acid corresponding to the side chain in the desired β-lactam antibiotic. In turn, this enables an increased flux through the step of the biosynthesis of the β-lactam in which the side chain is connected to the β-lactam nucleus and results in a reduction of the intermediate 6-APA and byproducts like 8-OH penicillic acid.
Said inducing culture conditions may be dependent on the promoter which is involved in directing expression of the ligase of the invention.

The advantages of the improved processes, according to the invention, is firstly, a process, according to the invention, which may result in a significantly elevated accumulated yield of the \(\beta\)-lactam antibiotic of interest.

Secondly, due to formation of less byproduct(s), the recovery and purification of \(\beta\)-lactam antibiotics, in substantially pure form, is facilitated.

Thirdly, the invention permits the production of the \(\beta\)-lactam antibiotics of interest, without the production of a significant amount of waste products. Consequently this makes the processes more efficient, due to more energy available to synthesize the products of interest. Further the waste-products may interfere adversely with the biosynthetic pathway.

Fourthly, the yield of the \(\beta\)-lactam antibiotic of interest is, at any time in the fermentation process, significantly elevated in comparison to fermentation processes with microorganisms lacking or having low activity of said ligase. In this context the yield may be an overall yield or the yield over a certain period of time.

All the above mentioned advantages make the processes of producing industrial important \(\beta\)-lactam antibiotics more efficient.

The expression of ligase activity or just an increase of said ligase activity, in microorganisms lacking or having low ligase activity, according to the invention, can make non-enzymatic removal of side-chains of \(\beta\)-lactam antibiotic intermediates superfluous in the production of e.g. penicillins and cephalosporins.

According to the invention it has been found that it is possible to produce certain \(\beta\)-lactam antibiotics using entirely enzymatically catalyzed processes, consequently without any use of chemical processes.

In an embodiment of the invention, an adipoyl-penicillin or an adipoylcephalosporin, such as adipoyl-7-ADCA or adipoyl-7-ACA is produced entirely, by enzymatic means, \textit{in vivo}, by inducing the host microorganism to express an increased ligase activity, according to the invention. Further present is an acyltransferase (AT) (e.g. the acyl-coenzyme A:isopenicillin N acyltransferase from \textit{P. chrysogenum}) and enzymes able to transform penicillins into cephalosporins (e.g. expandase from \textit{S. clavuligerus}).
Experimental
Detection of acyl-coenzyme A compounds

Equipment
5 HPLC-system (TSP)
   equipped with degasser, pump P4000, autosampler AS3000, detector
   UV2000, interface SN4000

Column: Supelcosil RP LC-18-DB with Supelguard
Detection: UV-Detection at 254 nm
10 Flow: 1 ml/min

Reference enzymes and compounds
Acetyl-coenzyme A (Sigma)
Acetyl-coenzyme A synthetase (Sigma)

Phenoxyacetic acid coenzyme A ester (M. J. Alonso et al., J. Antibiot., 41, p. 1074-
1084, 1988)
Phenylacetic acid coenzyme A ester (Sigma)

Adipic acid coenzyme A ester was prepared as follows: Adipoyl chloride (0.14 ml;
Aldrich) was added to a well-stirred and ice-water cooled suspension of
coenzyme A - Na salt (400 mg; 5.152 x 10^{-4} mmol; Sigma) in acetone
(60 ml; Merck PA) + water (0.6 ml) + 0.2M KHCO_{3} (to raise the pH to
> 7) under an atmosphere of nitrogen. The pH of the reaction contents
were brought to about 7.7 and, then, further stirred for about 60 min at
the same temperature. Thereafter, acetone was removed partially under
reduced pressure, the resulting product dissolved in cold water and freeze
dried. Yield 0.0890 g. Finally, the product was subjected to Ultrafiltration.

Solutions
Solution A: 10 \mu l of 0.25 M MgCl_{2}, 50 \mu l of 0.1 M ATP in 50 mM Tris/HCl, pH
8.0, 50 \mu l of 20 mM CoASH in 50 mM Tris/HCl, pH 8.0 and 30 \mu l
of 0.2 M phenoxyacetic acid or phenylacetic acid or adipic acid or
hexanoic acid in 50 mM Tris/HCl, pH 8.0 mixed and equilibrated
at 25^\circ C for 5 minutes.

Solution B: 100 \mu l of 0.25 M MgCl_{2}, 500 \mu l of 0.1 M ATP in 50 mM Tris/HCl,
500 \mu l of 20 mM CoASH in 50 mM Tris/HCl, and 300 \mu l of 0.067M
potassium acetate in 50 mM Tris/HCl.
Direct assay for phenyl- and phenoxyacetyl-coenzyme A synthetase activity

The reaction was started by addition of 100 μl of enzyme solution to Solution A containing phenylacetic or phenoxyacetic acid and CoASH. After incubation for 30 minutes at 25°C the reaction was stopped by addition of 240 μl of 0.5% trifluoroacetic acid. The precipitate formed was removed by centrifugation and the amount of phenoxyacetic acid coenzyme A ester (or phenylacetic acid coenzyme A ester) formed was analyzed by HPLC, eluent 15% (v/v) acetonitrile in 25 mM sodium phosphate buffer, pH 6.5.

Retention time for phenoxyacetic acid coenzyme A ester: 9.7 minutes and for phenylacetic acid coenzyme A ester: 9.0 minutes. Phenyl- and phenoxyacetic acid coenzyme A esters were quantitated relative to a standard.

Direct assay for adipoyl-coenzyme A synthetase activity

The reaction was started by addition of 1 volume of enzyme to 1.4 volumes of solution A containing adipic acid, adjusted to pH 8.5. A typical incubation mixture consisted in 250 μl enzyme and 350 μl solution A. Incubation was at 30°C. At regular time intervals samples were withdrawn from the incubation mixture and mixed with an equal part of 0.5% trifluoroacetic acid. The precipitate formed was removed by centrifugation. The amount of adipoyl-coenzyme A ester formed was analyzed by HPLC, eluent 5% acetonitrile in 25 mM sodium phosphate buffer pH 6.5.

Retention time for adipoyl-coenzyme A ester: 11.4 minutes.
Adipoyl-coenzyme A ester was used as reference.
Formation of adipoyl-coenzyme A derivatives was also analyzed in this assay.

Direct assay for hexanoyl-coenzyme A synthetase activity

The reaction was started by addition of 1 volume of enzyme to 1.4 volumes of solution A containing hexanoic acid, adjusted to pH 8.5. A typical incubation mixture consisted in 250 μl enzyme and 350 μl solution A. Incubation was at 30°C.

At regular time intervals samples were withdrawn from the incubation mixture and mixed with an equal part of 0.5% trifluoroacetic acid. The precipitate formed was removed by centrifugation. The amount of hexanoyl-coenzyme A ester formed was analyzed by HPLC, eluent 15% acetonitrile in 25 mM sodium phosphate buffer pH 6.5.

Retention time for hexanoyl-coenzyme A ester: 20.4 minutes.
Direct assay for acetyl-coenzyme A synthetase activity

Equipment
HPLC (Waters)
ABI 1000S photodiode array HPLC-detector (Applied Biosystems)
Flow-one/Beta serie 100 radioactivity detector (Radiomatic)
Yttrium silicate flow cell (Radiomatic)

Solution B was mixed and pH was adjusted to 8.0 with 4 M KOH. 40 μl of acetic acid-1-14C, sodium salt, (41.8 mCi/mmol, 1.0 mCi/ml) was added and the mixture was equilibrated at 35 °C.

Assay: 25 μl of enzyme and 35 μl of Solution B was mixed and incubated at 35 °C for 30 minutes. The reaction was stopped by addition of 60 μl of 0.5% trifluoroacetic acid. Any precipitate formed was removed by centrifugation and amount of acetyl-coenzyme A ester formed was analyzed by HPLC (eluent 5% (v/v) acetonitrile in 25 mM sodiumphosphate buffer pH 6.5). Radioactive compounds were detected using the Radiomatic detector equipped with a 250 μl yttrium silicate flow cell and non-radioactive compounds by UV detection at 210 nm with a detector placed in series with the Radiomatic detector.

Retention time for acetyl-coenzyme A ester: 6.9 minutes
As standard was used acetyl-coenzyme A (Sigma) as well as incubation of the substrate solution B with acetyl-coenzyme A synthetase (Sigma) followed by analysis of the formed 14C-acetyl-coenzyme A by HPLC.

Example 1
Fermentation of P. chrysogenum

A two-stage fermentation of the P. chrysogenum Wisconsin 54-1255 strain (ATCC 28089) in shake flasks was used for the production of β-Lactam compounds of interest or for the production of mycelia as a source of enzyme, RNA or DNA. The seed stage was initiated by adding 2 * 10⁸ spores to 50mL/500mL flask of medium composed of (g/L): glucose, 30; (NH₄)₂SO₄, 10; KH₂PO₄, 10; trace element solution I (MgSO₄·7H₂O, 25; FeSO₄·7H₂O, 10; CuSO₄·5H₂O, 0.5; ZnSO₄·7H₂O, 2; Na₂SO₄, 50; MnSO₄·H₂O, 2; CaCl₂·2H₂O, 5), 10 (mL/L) (pH before sterilization 6.5).

The seed culture is incubated for 48-72 hours at 25-30 °C and subsequently used to inoculate 10-20 volumes of a production medium containing (g/L) lactose, 80; maltose, 20; CaSO₄·4; urea, 3; MgSO₄·7H₂O, 2; KH₂PO₄, 7; NaCl, 0.5; NH₄NO₃, 6; FeSO₄·7H₂O, 0.1; trace element solution II (CuSO₄·5H₂O, 0.5;
ZnSO₄·7H₂O, 2; MnSO₄·H₂O, 2; Na₂SO₄, 50), 10 (mL/L); phenylacetic acid or adipic acid, 2.5% (w/v) (pH before sterilization 5.5-6.0). The incubation is then continued for another 96-120 hours.

Example 2
Partial purification of acyl-coA synthetases

Equipment
Phenyl-Sepharose CL 4B column (150 ml) (Pharmacia Biotech)
Pharmacia LKB PhastSystem (Pharmacia Biotech)

Solutions
Solution A: 50 mM Tris/HCl, pH 8.5; 35% ammonium sulphate, 4 mM EDTA, 5 mM ATP, 1 mM PMSF, 5 mM mercaptoethanol
Solution B: 50 mM Tris/HCl, pH 8.5, ammonium sulphate (20% saturation), 4 mM EDTA, 5 mM ATP, 5 mM ascorbic acid, 1 mM PMSF, 4 mM MgCl₂

Buffers
Buffer A: 50 mM Tris/HCl, pH 8.5, 1.36 M ammonium sulphate, 4 mM EDTA, 5 mM ATP, 5 mM ascorbic acid, 1 mM PMSF (phenyl-methyl-sulfonyl-fluoride)
Buffer B: 50 mM Tris/HCl, pH 8.5, 0.68 M ammonium sulphate, 4 mM EDTA, 5 mM ATP, 5 mM ascorbic acid, 1 mM PMSF
Buffer C: 50 mM Tris/HCl, pH 8.5, 20% glycerol, 4 mM EDTA, 5 mM ATP, 5 mM MgCl₂, 5 mM ascorbic acid, 1 mM PMSF

Harvest, extraction and precipitation
The cells from a 6 days old culture of P. chrysogenum (Example 1) were isolated by filtration and quickly washed with 5 volumes of 0.9% sodium chloride. The cells were frozen in liquid nitrogen and homogenized in a mortar. The enzyme was extracted by suspension in Solution A. Cell debris was removed by centrifugation. The extract was brought to 50 % ammonium sulphate saturation and the precipitate was removed by centrifugation. The enzyme could then be precipitated by addition of ammonium sulphate to 70 % saturation. After centrifugation the pellet was dissolved in Solution B.
Phenyl-Sepharose® CL 4B column chromatography

Approximately 2280 mg of protein (95 ml) (from the previous section) was applied to a Phenyl-Sepharose CL 4B column (150 ml), equilibrated in Buffer A, flow rate: 60 ml per hour. The column was washed with 500 ml of buffer A, followed by a gradient from 100 % Buffer B to 100 % Buffer C, total volume 1800 ml. Fractions of 8 ml were collected.

All fractions were analyzed for adipoyl- and phenylacetyl-coenzyme A synthetase activity and acetyl-coenzyme A synthetase activity (see Experimental section). The adipoyl- or phenylacetyl-coenzyme A synthetase activity eluted between the fractions Nos. 115-277, showing a peak of activity in fraction No. 175. An acetyl-coenzyme A synthetase activity eluted between the fractions Nos. 108-157, showing a peak of activity in fraction No. 118.

Example 3

Substrate specificity of three acyl-CoA synthetase preparations

Extraction, precipitation and partial purification of acyl-CoA synthetases from the P.chrysogenum Wisconsin 54-1255 strain cultured with either phenylacetic acid (PA) or adipic acid (AD) (Example 2), resulted in 2 different acyl-CoA synthetase preparations. A third acyl-CoA synthetase preparte was obtained by purification from cells obtained from a phenoxyacetic acid (POA) fermentation (see International patent application WO 96/10085).

The obtained partially purified acyl-coenzyme A synthetases were incubated with phenoxyacetic acid, phenylacetic acid, adipic acid, hexanoic acid and acetic acid, using the assay conditions given in the section Experimental. The substrate specificities which were obtained are depicted in Table 1 (relative activities are given).

Table 1: Substrate specificity of three acyl-CoA synthetase preparations as analyzed on different substrates

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Precursor in fermentation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>POA</td>
</tr>
<tr>
<td>phenoxyacetic acid</td>
<td>100</td>
</tr>
<tr>
<td>phenylacetic acid</td>
<td>8</td>
</tr>
<tr>
<td>adipic acid</td>
<td>22</td>
</tr>
<tr>
<td>hexanoic acid</td>
<td>295</td>
</tr>
<tr>
<td>acetic acid</td>
<td>&lt; 1</td>
</tr>
</tbody>
</table>
The enzyme preparation from a cell-free extract obtained by fermentation of *P. chrysogenum* in the presence of adipic acid displays a large increase in specificity towards adipic acid relative to the specificity towards other precursors.

**Example 4**

**Purification of adipoyl-CoA synthetase activity**

As shown in Example 3, an enzyme preparation obtained from mycelium grown in the presence of adipate had activity towards other sidechain precursors as well. An enzyme preparation from adipate-induced mycelium was subjected to further purification, as described below.

**Equipment**

- FPLC-system (Pharmacia)
- Mini-PROTEAN II Electrophoresis System (Bio-Rad)
- Sorvall centrifuge RC-5B

**Materials**

- Complete™ (Boehringer Mannheim)
- Ammonium sulfate for biochemistry (Merck)
- PD-10 column (Pharmacia)
- Triton X-100 (Merck)
- Bio Gel HT gel Hydroxyapatite (Bio-Rad)
- Reactive Green 19 agarose (Sigma)

**Harvest, extraction and precipitation**

**Buffers**

- **Buffer A:** 50 mM Tris/HCl pH 8.5, 1.36 M ammonium sulfate, 5 mM MgCl₂, 5 mM ATP, 4 mM EDTA, Complete™ protease inhibitor (1 tablet / 50 ml buffer), 5 mM β-mercaptoethanol
- **Buffer B:** 50 mM Tris/HCl pH 8.5, 0.68 ammonium sulfate, 5 mM MgCl₂, 5 mM ATP, 4 mM EDTA, Complete™ protease inhibitor (1 tablet / 50 ml buffer), 5 mM ascorbic acid

**Extraction**

The enzyme was extracted by suspension of lyophilized mycelium in buffer A (1 g / 20 ml) for 45 min on ice. Cell debris was removed by centrifugation (13,000
r.p.m. GSA-rotor, 30 min). The extract was brought to 45 % ammoniumsulfate saturation, precipitated on ice for 45 min, and the precipitate was removed bij centrifugation (13.000 r.p.m. GSA-rotor, 30 min). The enzyme could then be precipitated bij addition of ammonium sulfate to 65 % saturation, precipitation on ice for 45 min, and collection of the precipitate by centrifugation (13.000 r.p.m. GSA-rotor, 30 min).

After centrifugation the pellet was dissolved in buffer B.

**Triton X-100 extraction**

**Buffers**

PD-10 buffer: 10 mM Tris/HCl pH 8.5, 4 mM EDTA, 5 mM MgCl₂, 20% glycerol, 5 mM ATP, Complete™ protease inhibitor (1 tablet /50 ml buffer), 5 mM ascorbic acid

**Extraction**

Before extraction with detergent Triton X-100, protein from the previous section was desalted with a PD-10 column with PD-10 buffer according to the procedure provided bij Pharmacia.

The protein concentration was determined by Bradford.

20 % Triton X-100 was made in PD-10 buffer. 20% Triton X-100 solution was added dropwise to the protein solution, to a final concentration of 1% Triton X-100 and 10 mg/ml protein. Triton X-100 extraction was carried out for 1 hr on ice.

**Hydroxyapatite Chromatography (HIAB)**

**Buffers**

Buffer A: 5 mM potassium phosphate buffer pH 7.8, 5 Mm MgCl₂, 4 mM EDTA, 10% glycerol, 5 mM ascorbic acid, 5 mM ATP, Complete™

Buffer B: 200 mM potassium phosphate buffer pH 7.8, 5 Mm MgCl₂, 4 mM EDTA, 10% glycerol, 5 mM ascorbic acid, 5 mM ATP, Complete™

**Chromatography**

10 ml Triton X-100 extract (= 100 mg) was applied on a hydroxyapatite-column (volume 30 ml) which is equilibrated with buffer A. Flow was 0.5 ml/min. The column was washed with buffer A (approximately 120 ml). Protein was eluted
with buffer B. (≈ 30 mg). Fractions were analyzed for adipoylCoA synthetase activity.

**Reactive Green 19 Chromatography**

**Buffers**

Buffer A: 10 mM Tris/HCl pH 7.8, 2 mM MgCl₂, 20% glycerol, 5 mM ATP, 5 mM ascorbic acid, Complete™

Buffer B: 10 mM Tris/HCl pH 7.8, 2 mM MgCl₂, 20% glycerol, 5 mM ATP, 5 mM ascorbic acid, Complete™, 450 mM KCl

**Chromatography**

Active fractions from HIAB (approximately 5 mg) were applied on a Reactive Green 19-column (volume 5 ml) which was equilibrated with 50 ml Buffer A. Flow was 0.3 ml/min. The column was washed with buffer A (approximately 25 ml). Protein was eluted with buffer B according to the following gradient:

<table>
<thead>
<tr>
<th>Volume (ml)</th>
<th>% buffer B</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>50</td>
<td>80 (≈ 360 mM KCl)</td>
</tr>
<tr>
<td>70</td>
<td>80</td>
</tr>
</tbody>
</table>

Fractions of 2 ml were collected. Fractions were analyzed for adipoylCoA synthetase activity. Activity was measured in fractions 40-56 ml, showing a peak at 48 ml.

The synthetase activities with adipate, phenylacetate, and phenoxyacetate were measured in different fractions. As shown in Table 2, the ratio of adipoyl-CoA synthetase activity to phenylacetyl-CoA synthetase activity and phenoxyacetyl-CoA synthetase activity changed during the purification, favoring adipoyl-CoA synthetase activity. These data show that with this procedure adipoyl-CoA synthetase is separated from other enzymes, in particular phenylacetyl-CoA synthetase and phenoxyacetyl-CoA synthetase.
Table 2: CoA-synthetase activities with adipate or phenylacetate, in different fractions

<table>
<thead>
<tr>
<th>Fraction</th>
<th>CoA synthetase activity (arbitrary Units)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>phenoxacetate</td>
</tr>
<tr>
<td>Triton X-100 extract</td>
<td>nd</td>
</tr>
<tr>
<td>Hydroxyl Apatite</td>
<td>nd</td>
</tr>
<tr>
<td>Reactive Green</td>
<td>0</td>
</tr>
</tbody>
</table>

nd = not determined

Example 5

Identification of adipoyl-CoA synthetase from Penicillium

Equipment
Mini-PROTEAN II Electrophoresis System (Bio-Rad)
Stirrer

Materials
30 % (w/v) Acrylamide/ Bis solution (37.5:1) (GibCo BRL)
TEMED (LKB)
Broad Range marker (Bio-Rad)
Rainbow marker (Amersham)

A partially purified protein fraction containing adipoyl-CoA synthetase activity was subjected to chromatography on a native gel (Methods in Molecular Biology, Volume 32, p. 17-22, Basic Protein and Peptide protocols. J.M.Walker).

Two 7.5 % gels were prepared:

Electrophoresis buffer was chilled before use. The Mini-PROTEAN II Electrophoresis System was placed on icewater during electrophoresis. A stirrer bar was placed in the System to ensure proper mixing of the chilled water. Electrophoresis was performed at 30 mA until the bromophenol blue reached the bottom of the gel (approximately 2 hr).

After electrophoresis, the gel was allowed to equilibrate in a buffering solution (see below). To identify the adipoyl-CoA synthetase, an activity staining was carried out, based on the formation of pyrophosphate from ATP during the activation of adipate. The formation of pyrophosphate results in precipitation with Ca^{2+}, appearing as a white band in the gel. The following mixture for activity-staining was prepared in degassed 50 mM Tris/HCl, pH 8.5: 4 mM CoA, 5 mM ATP, 15
mM MgCl$_2$, 10 mM CaCl$_2$. Incubation took place for 10 min. bij gently shaking (the gel was completely covered with the mixture). Then, adipate was added to a final concentration of 10 mM, and the gel was further incubated without shaking.

The precipitate occurred within 30 min of incubation (no precipitate was visible without adipate). Adipoyl-CoA ligase activity was clearly present as a single band. The protein had an apparent mobility between 46 and 66 kD, based on the mobility of the marker proteins (Rainbow Marker, Amersham) on a native gel.

The gel fragment can be cut out to isolate the enzyme expressing the adipoyl-CoA synthetase activity and/or can be used for partial amino acid sequence determination.

**Example 6**

**Substrate specificity of adipoyl-CoA synthetase**

**Materials**
- adipate (Merck)
- carboxymethylthiopropionate (Fluka)
- phenylacetate (Sigma)
- phenoxyacetate (Sigma)
- trans-β-hydromuconate (Fluka)

An enzyme preparation of mycelium grown in the presence of adipic acid was subjected to several purification steps, including ammonium sulfate precipitation, hydroxyl apatite, and Reactive Green chromatography. A fraction that had a high specific CoA synthetase activity towards adipate, while lacking activity towards phenylacetate, was tested with different adipate derivatives, including carboxymethylthiopropionate (CMTP, as disclosed in WO 95/04148) and trans-β-hydromuconate. In Table 3, data are presented showing that adipoyl-CoA ligase has a substrate specificity which includes S-derivatives of adipate (CMTP) and other mono- and dicarboxylic acids (saturated or unsaturated).
Table 3: Substrate specificity of adipoyl-CoA synthetase

<table>
<thead>
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<th>Substrate</th>
<th>CoA synthetase activity</th>
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<tr>
<td>adipate</td>
<td>+++</td>
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<tr>
<td>carboxymethylthio-propionate</td>
<td>+</td>
</tr>
<tr>
<td>phenyl acetate</td>
<td>-</td>
</tr>
<tr>
<td>phenoxy acetate</td>
<td>-</td>
</tr>
<tr>
<td>trans-β-hydromuconate</td>
<td>+</td>
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</table>
Claims

1. An enzyme obtainable from *Penicillium chrysogenum* catalyzing the formation of an acyl-coenzyme A being an adipoyl-coenzyme A.

2. The enzyme of claim 1, which further catalyzes the formation of adipoyl-coenzyme A derivatives such as carboxymethylthiopropionyl-coenzyme A and trans-\(\beta\)-hydromuconyl-coenzyme A.

3. The enzyme of claim 1 or 2, which is at least between 10 and 100 times, preferably 10\(^3\) times and more preferably between 10\(^4\) and 10\(^6\) times more active towards adipic acid than towards acetic acid or than towards phenylacetic or phenoxyacetic acid.

4. A DNA fragment comprising a DNA sequence encoding the enzyme of any one of the claims 1 to 3, said DNA sequence being obtainable from *Penicillium chrysogenum*.

5. An expression cassette comprising the DNA sequence as defined in claim 4.

6. The expression cassette of claim 5, wherein said DNA sequence is operably linked to a promoter sequence and optionally to a terminator sequence.

7. The expression cassette of claim 6, wherein said promoter sequence is from another gene involved in the biosynthesis of \(\beta\)-lactams than from the gene encoding the ligase.

8. The expression cassette of claim 5, wherein said DNA sequence is operably linked to a sequence encoding a targeting signal or secretion signal.

9. A vector or transformation vehicle comprising the expression cassette of any one of the claims 5 to 8.

10. A microbial host cell comprising the expression cassette of any one of the claims 5 to 8 or the vector or transformation vehicle of claim 9.
11. The microbial host cell of claim 10, which is a bacterial or a fungal host cell.

12. The microbial host cell of claim 11, wherein the bacterial host is a gram-positive bacterium, e.g. belonging to the genus *Bacillus* or *Streptomyces* or a gram-negative bacterium, e.g. belonging to the genus *Escherichia*, and the fungal host is a filamentous fungus, e.g. belonging to the genus *Aspergillus*, *Acremonium* or *Penicillium*.

13. A process for the production of a β-lactam antibiotic comprising the use of an increased adipoyl-coenzyme A ligase activity.

14. A process for the production of a β-lactam antibiotic comprising
   i) fermentation of a microorganism capable of producing said β-lactam antibiotic
   which is transformed with the expression cassette of any one of the claims 5 to 8 or the vector or transformation vehicle of claim 9, and
   ii) recovering said β-lactam antibiotic.

15. The process of claim 14, wherein the transformed microorganism is from the group comprising *Penicillium*, *Acremonium*, *Aspergillus*, *Nocardia*, *Streptomyces*, *Bacillus*, *Ceroplasta*, *Microspora*, other *Eubacteria*, other *Actinomycetes* or filamentous fungi.

16. The process of claim 15, wherein the transformed microorganism belongs to species from the group comprising *Penicillium chrysogenum*, *Penicillium notatum*, *Acremonium chrysogenum*, *Aspergillus nidulans*, *Nocardia lactamdurus* and *Streptomyces clavuligerus*. 
INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N15/31 C12N15/52 C12N9/00 C12P35/00 C12N1/15
C12N1/21 C12N15/80 C12P37/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N C12P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic database consulted during the international search (name of database and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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<td>BIO/TECHNOLOGY, vol. 13, January 1995, pages 58-62, XP002037169 CRAWFORD, L., ET AL.: &quot;PRODUCTION OF CEPHALOSPORIN INTERMEDIATES BY FEEDING ADIPIC ACID TO RECOMBINANT PENICILLIUM CHRYSOGENUM STRAINS EXPRESSING RING EXPANSION ACTIVITY&quot; cited in the application especially page 60, right column, line 11-15 see the whole document</td>
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Further documents are listed in the continuation of box C. Patent family members are listed in annex.

Date of the actual completion of the international search

7 August 1997

Date of mailing of the international search report

20 08 97

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HT Rijswijk
Tel. (+31-70) 340-2040, Tx. 31 651 eipo nl, Fax (+31-70) 340-2016

Authorized officer

Holtorf, S
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